Silymarin component 2,3-dehydrosilybin attenuates cardiomyocyte damage following hypoxia/reoxygenation by limiting oxidative stress

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Short title: 2,3-dehydrosilybin attenuates cardiomyocyte damage.

Summary

Ischemic postconditioning and remote conditioning are potentially useful tools for protecting ischemic myocardium. This study tested the hypothesis that 2,3-dehydrosilybin (DHS), a flavonolignan component of *Silybum marianum*, could attenuate cardiomyocyte damage following hypoxia/reoxygenation by decreasing the generation of reactive oxygen species (ROS). After 5-6 days of cell culture in normoxic conditions the rat neonatal cardiomyocytes were divided into four groups. Control group (9 h at normoxic conditions), hypoxia/reoxygenation group (3 h at 1 % O_2, 94 % N_2 and 5 % CO_2 followed by 10 min of 10 $\mu mol \cdot l^{-1}$ DHS and 6 h of reoxygenation in normoxia) and postconditioning group (3 h of hypoxia, three cycles of 5 min reoxygenation and 5 min hypoxia followed by 6 h of normoxia). Cell viability assessed by propidium iodide staining was decreased after DHS treatment consistent with increased levels of lactatedehydrogenase (LDH) after reoxygenation. LDH leakage was significantly reduced when cardiomyocytes in the H/Re group were exposed to DHS. DHS treatment reduced H_2O_2 production and also decreased the generation of ROS in the H/Re group as evidenced by a fluorescence indicator. DHS treatment reduces reoxygenation-induced injury in cardiomyocytes by attenuation of ROS generation, H_2O_2 and protein carbonyls levels. In addition, we found that both the postconditioning protocol and the DHS treatment are associated with restored ratio of phosphorylated/total protein kinase C epsilon, relative to the H/Re group. In conclusion, our data support the protective role of DHS in hypoxia/reperfusion injury and indicate that DHS may act as a postconditioning mimic.

Key words: silymarin, dehydrosilybin; neonatal rat cardiomyocytes; postconditioning; reactive oxygen species

1. Introduction

Silymarin, a seed extract from milk thistle plant *Silybum marianum*, has been used to treat a range of liver and gallbladder disorders, including hepatitis and cirrhosis, and to protect the liver against poisonings from chemical and environmental toxins (Rambaldi *et al.* 2005). The effects of silymarin, including its effect on cell signalling pathways, its pharmacokinetics, pharmacodynamics, tissue distribution, bioavailability and safety in humans, summarized by Agarwal (Agarwal *et al.* 2006) and others (Kren *et al.*). Other studies have demonstrated that silymarin has cardioprotective activity in ischemia-reperfusion-induced myocardial infarction (Rao and Viswanath 2007) and oxidative stress in mice and rat cardiac tissues (Nabavi *et al.* 2012, Taghiabadi *et al.* 2012).

The silymarin mixture contains several major (Ding *et al.* 2001) as well as minor polyphenolic compounds (Kim *et al.* 2003). The flavonolignans silybin, isosilybin, silychristin, silydianin, 2,3-dehydrosilybin (DHS), flavonoid taxifolin and polymeric polyphenolic compounds are the main active compounds that have interesting and important therapeutic activities. Silybin (SB) represents more than 50 % of the silymarin mixture and is generally thought to be the most therapeutically active component (Comelli *et al.* 2007). However, it was suggested that not the major flavonolignans, but the minor components are responsible for the antioxidant capacity of the silymarin mixture (Gabrielova *et al.* 2010, Kvasnicka *et al.* 2003, Weidmann 2012).

Ischemia/reperfusion (I/R) injury is characterized by significant oxidative stress, which induces characteristic changes in the antioxidant system and organ injury leading to significant morbidity and mortality. Cardiomyocytes can resist ischemia/reperfusion injury through ischemic postconditioning, i.e. a repetitive ischemia induced during the onset of reperfusion (Hausenloy and Yellon 2008, Vinten-Johansen 2007, Zhao *et al.* 2003). Ischemic postconditioning and remote conditioning are considered beneficial based on small clinical

trials (Garcia *et al.* 2010, Lonborg *et al.* 2010, Thibault *et al.* 2008). Postconditioning was shown to reduce hypoxia/reoxygenation-induced cardiomyocyte necrosis and apoptosis (Sun *et al.* 2005, Wang *et al.* 2006).

Generation of reactive oxygen species (ROS) is a well established cause of heart injury induced by ischemia-reperfusion (Becker *et al.* 1999, Vanden Hoek *et al.* 1997). Mitochondria-generated superoxide anion and subsequently other ROS, such as hydrogen peroxide and hydroxyl radical, can compromise cell viability because of damage to proteins, lipids and nucleic acids. Under physiological conditions, cellular damage is diminished by antioxidant defences that neutralize ROS (Cadenas 1989). It was suggested that depending on their structures and concentrations antioxidants are able to protect against cardiovascular diseases (Guaiquil *et al.* 2004), and interest in the use of natural and synthetic antioxidants as functional food ingredients or as food supplements increases (Celik and Arinc 2010, Puertollano *et al.* 2011).

Recent research has pointed to receptors, kinase signalling pathways and mitochondrial participation in ischemic conditioning (Buchholz *et al.* 2014, Garlid *et al.* 2009). Pharmacological postconditioning represents administration of pharmacological agents at the time of reperfusion after a prolonged period of ischemia (Zhao, Corvera 2003, Zhao 2010) and modifications of the cardioprotective cell signalling pathways by pharmacological agents mimics the cardioprotection by ischemic postconditioning (Buchholz, Donato 2014). Several pharmacological agents possess the ability of mimicking the effects of ischemic conditioning and can also reduce the number of cells dying during ischemia/reperfusion (Wang *et al.* 2011, Ye *et al.* 2011). Recent studies have shown that pools of various kinases, including Akt and protein kinase C epsilon (PKCε), are localized to mitochondria and play a critical role in cardioprotection (Garlid, Costa 2009, Miura *et al.* 2010).

In the present study, we tested the hypothesis that DHS could attenuate cardiomyocyte damage following hypoxia/reoxygenation by decreasing the generation of ROS. Using rat neonatal cardiomyocytes, we observed a significant decrease of hypoxia/reoxygenation injury in cardiomyocytes treated with a single dose of DHS. In addition, we found that both the postconditioning protocol and the administration of DHS are associated with restored ratio of phosphorylated/total PKCε. In conclusion, our data support the protective role of DHS in hypoxia/reperfusion injury and indicate that DHS may act as a postconditioning mimic.

2. Methods

2.1 Chemicals and test compounds

Dulbecco's modified Eagle's medium (DMEM), heat-inactivated fetal bovine serum (FBS), stabilized penicillin-streptomycin solution (PenStrep), sterile dimethylsulfoxide (DMSO), propidium iodide (PI), horseradish peroxidase (HRP), β-Nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH) and all other chemicals were obtained from Sigma-Aldrich. Dihydrorhodamine 123 (DHR) was from Fluka. Amplex red was from Life Technologies.

2,3-dehydrosilybin (DHS, 98%; $C_{25}H_{20}O_{10}$, Mr 480) was prepared at the Institute of Microbiology, Academy of Science of the Czech Republic, Prague, Czech Republic. The method of DHS preparation is based on base-catalyzed disproportionation (Gažák *et al.* 2013). Silybin (2.5 g, 5.183 mmol) and $NaHCO_3$ (1.74 g, 20.798 mmol) were dissolved in methanol (100 ml) and the mixture was heated under reflux for 16 h. The mixture was then left to cool to room temperature and poured into ice-cold water containing HCl (400 ml, 5% v/v). The precipitate formed was filtered off, washed with H_2O, dissolved in a mixture of ethyl acetate/acetone (1:1), and evaporated to give 2.17 g of dry residue. The solid was crystallized from methanol (1000 mg, 40% yield). The mother liquor was filtered through a silica gel pad

($CHCl_3$/acetone/HCOOH 90:10:1–70:30:1) to obtain, after concentration, another portion of the product, which after recrystallization from methanol yielded pure DHS (270 mg, 11%). Thus, the total yield of DHS was 51%. Stock solution of DHS (10 $mmol \cdot l^{-1}$) was prepared in DMSO. The final concentration of DMSO in the medium was 0.5% (v/v).

2.2 Animals

Wistar rats (250 to 275 g) were bred and housed in a certified animal house according to EU rules and according to the Faculty of Medicine and Dentistry, Palacky University, in accordance with the Guide for the Care and Use of Laboratory Animals (1985), NIH, Bethesda, or European Guidelines on Laboratory Animal Care. Animal treatment and sacrifice procedure for neonatal rats was approved by the Ethical Committee for Laboratory Animal Treatment of the Faculty of Medicine and Dentistry, Palacky University.

2.3 Isolation of neonatal rat cardiomyocytes

The procedure described by Chlopcikova et al. (Chlopcikova *et al.* 2001) was followed. Entire hearts were isolated from 2 - 5 day old rats and minced in a balanced salt solution containing in $mmol \cdot l^{-1}$: 20 HEPES, 120 NaCl, 1 NaH_2PO_4, 5.5 glucose, 5.4 KCl and 0.8 $MgSO_4$ (pH 7.3 - 7.4). The consequent trypsin digestion released cells that were resuspended in a medium containing Dulbecco's Modified Eagle Medium (DMEM) and a medium 199 (4:1) supplemented with horse serum (10%), fetal calf serum (5%), penicillin (100 $U \cdot ml^{-1}$) and streptomycin (100 $\mu g \cdot ml^{-1}$). The suspension enriched in non-adhesive myocytes was transferred to collagen I-coated culture dishes at a density of 5×10^4 cells per cm^2. The cells were incubated in 95% air and 5% CO_2 at 37 °C. The medium was removed after 72 h and replaced with a culture medium containing DMEM and medium 199 (4:1) with penicillin (100 $U \cdot ml^{-1}$) and streptomycin (100 $\mu g \cdot ml^{-1}$). Cultured cardiomyocytes were allowed to reach

a confluence before being used experimentally. The percentage of beating myocardial cells exceeded 85 - 90 % after 3 days in culture for each experiment.

We used commercial real-time xCELLigence RTCA system based on impedance measurement on microtiter plates that are integrated with gold microelectrodes (Ke *et al.* 2011), to determine the best cellular density conditions for our model. We monitored the viability profile of rat neonatal cardiomyocytes in real-time, thereby determining the optimal time for starting the hypoxia/reoxygenation experiments. Based on the data we subjected the cell culture to the hypoxia/reoxygenation treatment protocol within the time frame of 72-120 h after isolation and seeding (data not shown).

2.4 Hypoxia/reoxygenation treatment protocol

We used in vitro protocol for the cell culture model of hypoxia postconditioning according to an established procedure (Sun, Wang 2005, Sun *et al.* 2006, Tang *et al.* 2014, Tu *et al.* 2012). After 5 - 6 days of cell culture in normoxic 10% FBS-DMEM, the culture medium was freshly changed with 1% FBS-DMEM, and the cardiomyocytes were divided into three groups as follows: 1. control, in which cardiomyocytes were incubated for 9 h at normoxic conditions (Fig. 1A); 2. hypoxia/reoxygenation (H/Re), in which the culture dishes were transferred to a hypoxic incubator, atmosphere of 1 % O_2, 94 % N_2 and 5 % CO_2 for 3 h followed by 6 h of reoxygenation (Fig. 1B); 3. Postconditioning (PostC), in which cardiomyocytes were postconditioned after the 3 h index hypoxia by three cycles of 5 min of reoxygenation and 5 min of rehypoxia. After the three cycles of postconditioning 6 h of continuous normoxia was followed (Fig. 1C); 4. hypoxia/reoxygenation (H/Re) + DHS, in which the culture dishes were transferred to a hypoxic incubator, atmosphere of 1 % O_2, 94 % N_2 and 5 % CO_2 for 3 h followed by 10 min of 10 $\mu mol \cdot l^{-1}$ 2,3-dehydrosilybin and 6 h of reoxygenation (Fig. 1D). The incubator utilized for hypoxia experiments is equipped with O_2

(Envitec OOM202-2) and CO_2 (infrared diode) sensors allowing continuous monitoring of gas composition (Mezentech, Czech Republic). It maintains optimal temperature and variable gas concentration for *in vitro* cultivation of cells in various types of cultivation vessels (software Machine Code for AT-MEGA microprocessor).

2.5 Identification and quantification of cell death by flow cytometry

Harvested cardiomyocytes were stained for 30 min in the dark with 1 ml of Vindal's solution that consisted of 20 $\mu g \cdot ml^{-1}$ RNase A, 20 $\mu g \cdot ml^{-1}$ propidium iodide (PI) in the presence of 0.1% Triton X-100 to quantify total cell death. 0.5 ml of the stained cardiomyocytes were mixed with 0.5 ml of Vindal's solution and were analyzed by flow cytometry on Cytomics FC 500 (Beckman Coulter, Fullerton, CA, USA) as described (Vrba *et al.* 2009), counting 10,000 events per sample. The percentage of cells in sub-G1 peak was calculated using MultiCycle AV Software (Phoenix Flow Systems, San Diego, CA, USA) which eliminated the debris effect. Each experiment was performed in triplicate and independently repeated at least four times.

2.6 Lactate dehydrogenase release assay

The activity of lactate dehydrogenase (LDH) released into the culture medium via cell membrane disruption was measured spectrophotometrically. The method is based on LDH-catalysed reduction of pyruvate to lactate by an equimolar amount of NADH, the decrease of which was monitored at 340 nm (Maines 1998). After incubation, serum-free medium (50 µl) was mixed with 150 µl of fresh LDH buffer (50 $mmol \cdot l^{-1}$ Na_2HPO_4; 1.22 $mmol \cdot l^{-1}$ sodium pyruvate; pH 7.5) containing NADH (0.465 $mmol \cdot l^{-1}$) on a microplate and absorbance change over time (ΔA/min) was monitored (Tecan Infinite M200, Tecan, Austria). The viability of treated cells was expressed in $nkat \cdot l^{-1}$. Protein concentration was estimated using the

Coommassie Blue method (Bradford 1976). Each experiment was performed in triplicate and independently repeated at least six times.

2.7 Measurement of cellular oxidative stress

The dihydrorhodamine 123 fluorescent probe (DHR, Molecular Probes) is converted into the fluorescent product rhodamine 123 by an interaction with H_2O_2 in the presence of peroxidase, cytochrome *c* or Fe^{2+} (Wardman 2007) and reacts also with peroxynitrite (Wardman 2008), making it a useful indicator of cellular oxidative stress. The fluorescence was monitored at specific excitation/emission wavelengths 488/525 nm. Cells were incubated with DHR (5 $nmol \cdot l^{-1}$) for 15 min in the dark, then washed once with PBS, scraped into PBS and sonicated to release the formed rhodamine 123. After centrifugation (2,000×*g*, 10 min, 4 °C), the fluorescence in the supernatant was measured using a microplate reader (Tecan Infinite M200, Tecan, Austria). Protein concentration was estimated using the Coommassie Blue method (Bradford 1976). The results were expressed as fluorescence of DHR per miligram of proteins. Each experiment was performed in triplicate and independently repeated at least six times.

2.8 Measurement of H_2O_2 production

The H_2O_2 production from cardiomyocytes was determined using the oxidation of the fluorogenic indicator Amplex red in the presence of horseradish peroxidase (HRP) (Zhou *et al.* 1997). In the presence of peroxidase Amplex red reagent reacts with hydrogen peroxide in a 1:1 stoichiometry to produce the red fluorescent oxidation product resorufin. Briefly, cardiomyocytes were initially lysed by three cycles of freeze-thawing, which was followed by centrifugation at 3,000×*g* for 10 min at 4 °C. 50 µl of supernatant was reacted with 50 µl of working solution containing 100 $\mu mol \cdot l^{-1}$ Amplex red (Invitrogen) reagent and 0.2 $U \cdot ml^{-1}$

HRP (Sigma Aldrich) in a 1× reaction buffer (0.05 $mol \cdot l^{-1}$ Na_2HPO_4, pH 7.4) and was incubated at room temperature for 30 min in the dark. Fluorescence was monitored on a reader (Tecan Infinite M200, Tecan, Austria) with excitation at 570 nm and emission at 585 nm. Micromoles of H_2O_2 were calculated using a standard curve generated from the readings of various concentrations of H_2O_2 (0 - 10 $\mu mol \cdot l^{-1}$). The results were expressed as micromoles per milligram of protein. Each experiment was performed in triplicate and independently repeated at least six times.

2.9 FTSC fluorometric assay of protein carbonyls

Fluorescein 5-thiosemicarbazide (FTSC) specifically reacts with carbonyl groups in oxidized proteins and not in oxidized lipids. Cells were washed once with PBS and scraped into PBS (100 µl). 50 µl samples were reacted with an equal volume of 0.2 $mmol \cdot l^{-1}$ FTSC overnight in the dark. Proteins were precipitated by the addition of 4 volumes of ice-cold 20% TCA (400 µl). Following 10 min of incubation on ice, tubes were centrifuged at 12,000×*g* for 10 min at 4 °C. Supernatants were carefully decanted; precipitates were washed three times by vortexing with 1 ml of aceton and centrifuged immediately. Finally, acetone supernatant was carefully decanted and protein precipitates were air-dried, solubilized with 50 µl of 6 $mol \cdot l^{-1}$ Guanidyl hydrochloride, and diluted 10-fold by the addition of 450 µl of 0.1 $mol \cdot l^{-1}$ NaH_2PO_4 (pH 7.0). Protein concentration in each of these samples was measured by the BCA assay, which requires that guanidyl hydrochloride be less than 4 $mol \cdot l^{-1}$. The samples were aliquoted 100 µl per well in triplicate into microtiter plate, and fluorescence was measured in a reader (Tecan Infinite M200, Tecan, Austria) with excitation at 485 nm and emission at 535 nm. Fluorescence readings from six wells for each sample were averaged, and nanomoles of FTSC-reacted carbonyls were calculated using a standard curve generated from the readings of various concentrations of FTSC prepared in medium similar to that of samples

(Mohanty *et al.* 2010). The results were expressed as micromoles per milligram of protein. Each experiment was performed in triplicate and independently repeated at least six times.

2.10 Western blotting analysis

Total proteins were prepared from rat neonatal cardiomyocytes in RIPA buffer (in $mmol \cdot l^{-1}$: 150 NaCl, 10 Tris, 5 EDTA, 0,1 Na_3VO_4, 1 NaF, 1% sodium deoxycholate, 10% SDS, 1% Triton X-100, 1 protease inhibitor cocktail tablet) and incubated at 4 °C for 30 min. The extracts were later centrifuged at 12 000 rpm at 4 °C for 15min. Equal amounts of total protein (20 μg) were boiled and separated with SDS-PAGE and electophoretically transferred to a PVDF membrane. Membranes were blocked with Tris-buffered saline-Tween 20 buffer (TBST) containing 5% non-fat milk at room temperature for 2 h, and then incubated overnight at 4 °C with primary antibodies. The primary antibody dilutions were 1:2000 for Actin (goat, sc-1616, Santa Cruz), 1:500 for p-PKCε Ser 729 (goat, sc-12355, Santa Cruz) and 1:500 for PKCε C-15 (rabbit, sc-214, Santa Cruz). The membrane was then washed three times with TBST buffer and incubated in TBST buffer containing 5% non-fat milk with secondary antibody (diluted 1:5000, Santa Cruz) for 1 h at room temperature. Finally, the membrane was washed with TBST for three times. For detection, the membranes were saturated with an enhanced chemiluminescence mixture for 1 min and viewed by autography using preflashed X-ray film (Kodak Scientific Imaging film) for 60 s. The bands were analysed by densitometric scanning using Typhoon 9400 scanner.

2.11 Statistics

Analysis of variance with post-hoc Tukey test was applied using STATISTICA software (StatSoft). $p < 0.05$ was considered statistically significant.

3. Results

3.1 DHS modulates cardiomyocyte viability and LDH release

We monitored cell viability by flow cytometry and LDH activity in the culture medium to evaluate the general effects of hypoxia and reoxygenation on neonatal rat cardiomyocytes. Hypoxia/reoxygenation significantly increased the number of dead cardiomyocytes as evidenced by an elevated peak of sub-G1 fraction (H/ Re vs ctrl group, $^{*}p < 0.001$) compared with the control group (Fig. 2). When cardiomyocytes were treated at the onset of reoxygenation with DHS (10 $\mu mol \cdot l^{-1}$, 10 min) the number of dead cardiomyocytes was reduced compared with the hypoxia/reoxygenation group (DHS vs H/Re group, $^{\dagger}p < 0.001$). The dose and exposure time for DHS treatment were selected based on our previous studies, which revealed the ability of DHS to suppress ROS formation (Gabrielova, Jaburek 2010). Pilot experiments verified that lower concentrations of DHS display the same protective activity in dose-dependent fashion but were not statistically significant.

LDH activity in the culture medium was used as an indicator of cytotoxicity. A significant increase of LDH released (H/Re vs ctrl group; $^{*}p < 0.001$) was detected in the hypoxia/reoxygenation group versus the control group (Fig. 3). LDH leakage in the medium was reduced when cardiomyocytes were postconditioned before full reoxygenation (PostC vs H/Re group, $^{\dagger}p < 0.001$). DHS treatment significantly diminished the H/Re-induced LDH release (DHS vs H/Re group, $^{\dagger}p < 0.001$; Fig. 3).

3.2 DHS attenuates production of reactive oxygen and nitrogen species

Reoxygenation is associated with elevated ROS formation therefore we employed an oxidation-sensitive fluorescent probe dihydrorhodamine to assess changes in intracellular ROS and reactive nitrogen species (RNS) levels. Cellular oxidative stress was increased as a result of hypoxia/reoxygenation treatment (H/Re vs ctrl group, $^{*}p < 0.001$) in comparison with

the postconditioned cardiomyocytes (PostC vs H/Re group, $^{\dagger}p < 0.001$). The increase of ROS/RNS formation was significantly diminished by 10 $\mu mol \cdot l^{-1}$ DHS (DHS vs H/Re group, $^{\dagger}p < 0.001$; Fig. 4).

3.3 DHS diminishes formation of hydrogen peroxide

Although a variety of ROS are present in a cell, hydrogen peroxide, formed by superoxide dismutase inside and outside of mitochondria, is of particular interest as it can diffuse through cellular membranes and interact with various cellular compartments. The H_2O_2 generation was detectable after 9 h of incubation in the control group but was significantly increased in hypoxic/reoxygenated cardiomyocytes relative to control cells (H/Re vs ctrl group, $^{*}p < 0.001$; Fig. 5). The level of hydrogen peroxide after 6 h of reoxygenation was significantly decreased in the group of cardiomyocytes treated with three cycles of postconditioning (PostC vs H/Re group, $^{\dagger}p < 0.001$) with a similar effect present in the cardiomyocytes treated with 10 $\mu mol \cdot l^{-1}$ DHS for 10 min (DHS vs H/Re group, $^{\dagger}p < 0.001$).

3.4 DHS decreases formation of protein carbonyls

The detected increase in ROS formation indicates a potential oxidative damage to the cell while measurement of selected markers of oxidative stress indicates the target of ROS-mediated damage. The detection of protein carbonylation is frequently used as a marker of irreversible protein oxidative modification. Recent reports (Fujita *et al.* 2007, Chaudhuri *et al.* 2006) have used a highly fluorescent compound, fluorescein 5-thiosemicarbazide (FTSC) that specifically reacts with carbonyl groups in oxidized proteins and not in oxidized lipids. A semi-microplate format assay of protein carbonyls using this FTSC-carbonyl reaction was developed (Mohanty, Bhamidipaty 2010). It measures directly the protein carbonyls similarly to the classical spectrophotometric assay and gives results comparable to the latter method. A

significant increase of carbonyl groups (H/Re vs ctrl group, $^{*}p < 0.001$) in oxidized proteins was detected in the hypoxia/reoxygenation group (Fig. 6). The concentration of protein carbonyls was reduced (PostC vs H/Re group, $^{\dagger}p < 0.001$) when cardiomyocytes were postconditioned before full reoxygenation. 10 $\mu mol \cdot l^{-1}$ DHS treatment after hypoxia also significantly inhibited H/Re-induced increase of protein carbonyls (DHS vs H/Re, $^{\dagger}p < 0.001$).

3.5 DHS restores the phosphorylated/total PKCε ratio

To investigate whether PKCε was involved in DHS-induced cardioprotection, the levels of PKCε were evaluated using western blotting analysis. Because one of the active PKCε is phosphorylated on Ser 729, we detected both total and phosphorylated PKCε (p-PKCε) in control, H/Re, PostC and DHS-treated (10 $\mu mol \cdot l^{-1}$, 10 min) groups using general and Ser 729 phosphospecific antibodies (Fig. 7A). The intensity of each band was quantified by densitometry and the ratio of pPKCε/PKCε was calculated from the data obtained by scanning and then normalized to the actin signal (Fig. 7B). The data show a significant decrease in the pPKCε/PKCε ratio in the hypoxia/reoxygenation group compared to the control (H/Re vs control group, $^{*}p < 0.001$) while both the postconditioning protocol and the DHS treatment significantly increased the pPKCε/PKCε ratio relative to the H/Re group (PostC vs H/Re group, DHS vs H/Re group, $^{\dagger}p < 0.001$) and restored the pPKCε/PKCε ratio to the levels of the control.

4. Discussion

Ischemia/reperfusion is the culprit in the eventual fatal damage to the heart muscle. Although cells are capable of adapting to lower oxygen conditions, adaptive mechanisms to deal with sudden reoxygenation are rather slow. Preventing the ischemic conditions is therefore desirable. However, it is very difficult to achieve, as in majority of cases we only find out about the occurrence after. Both postconditioning and pharmacological postconditioning aim at minimizing the damage arising from the index ischemia.

Postconditioning denotes a conditioning in which a decrease in the infarct size results from brief periods of ischemia applied during reperfusion, immediately following an ischemic insult (Hausenloy and Yellon 2008, Vinten-Johansen 2007). Postconditioning may reduce apoptosis, necrosis, and endothelial dysfunction/activation, thus leading to a reduced endothelium/leukocyte interaction and to reduced ROS inflammatory formation (Skyschally *et al.* 2009, Vinten-Johansen *et al.* 2010). Mechanisms of protection by ischaemic postconditioning have been studied in detail in the mammalian heart, in vitro and in vivo, and a number of themes have emerged. There is the involvement of endogenous cell-surface ligands such as adenosine, bradykinin, and opioids, which trigger protection by activating multiple intermediate kinase pathways. In fact, both preconditioning and postconditioning induce activation of signalling elements during the early reperfusion following the index ischemia (For review see Garlid et al. (Garlid, Costa 2009)). Pharmacological postconditioning involves the application of a pharmacological agent as soon as possible following the hypoxia period. Therefore, studies focused on the development of novel agents with protective activities against hypoxia/reoxygenation damage are of great interest.

Natural polyphenolic compounds display various cytoprotective activities and have been linked to cardioprotection (Fraga *et al.* 2010). Recent experimental data demonstrate that natural polyphenols can exert its cardioprotective effect via the activation of several

prosurvival cellular pathways (Lecour and Lamont 2011), however a definitive understanding of the mechanisms behind the cardioprotective effect of polyphenols is still a matter of ongoing investigation.

Our previous study evaluated cardioprotective effects of SB, DHS, and some of their derivatives and demonstrated that DHS displays activity in cardiomyocytes similar to that of synthetic uncouplers of oxidative phosphorylation. We have demonstrated that DHS attenuates the production of ROS in cardiomyocyte mitochondria by a mechanism which involves the uncoupling of the mitochondrial protonmotive force from the synthesis of ATP (Gabrielova, Jaburek 2010). We inferred that DHS protects cardiomyocytes against ROS-mediated damage thanks to its uncoupler-like behaviour (Gabrielova, Jaburek 2010). Our working hypothesis states that this very behaviour may assist the cells in dealing with the increase in oxygen inflow following reoxygenation because it triggers a demand for oxygen while keeping ROS formation at or close to physiological levels thus maintaining the signalling role of ROS.

Here we demonstrate that a brief treatment with a single dose of DHS has beneficial effect in the model of rat neonatal cardiomyocytes when cell death resulting from hypoxia/reoxygenation is diminished with concurrent modulation of ROS and ROS-dependent damage. Because DHS decreases mitochondrial ROS formation while increasing oxygen consumption accompanied by mitochondrial membrane potential decrease (Gabrielova, Jaburek 2010)), we questioned whether DHS may be capable of preventing oxidative damage due to reoxygenation in comparison with postconditioning. The evidence we present supports the role of DHS as a postconditioning mimic because in all aspects we evaluated, DHS showed the same or a very similar effect as postconditioning. The important aspect of DHS treatment is the time frame, where only ten minutes of treatment were sufficient to evoke an effect mimicking that of postconditioning.

DHS, a silybin derivative, was originally isolated as a minor component of the extract from seeds of the *Silybum marianum* subsp. *anatolicum*. This flavonolignan has multiple biological properties connected with its radical scavenging activity (Gazak *et al.* 2009). Although the radical scavenging ability and pharmacology of silybin (SB) were studied extensively, the molecular mechanisms of the antioxidant activity of SB and DHS were not systematically investigated and remain unclear. Two studies investigated the role of individual hydroxyl groups in SB and DHS in antiradical activity (Gazak, Sedmera 2009, Trouillas *et al.* 2008). In this case selectively methylated derivatives of SB and DHS were prepared and tested for their ability to interact directly with a radical. It was determined that C-20 hydroxyl group in SB and C-3 and C-20 hydroxyl groups of DHS are responsible for the interaction with radicals. However, direct interaction of the flavonolignans with radicals need not be the only or even the decisive mechanism of cytoprotective activity resulting in ROS modulation.

Here we show that DSH is very effective in ROS modulation, hence preventing hypoxia/reoxygenation damage in rat neonatal cardiomyocytes. The importance of a reduced endothelial activation, neutrophil adherence and consequently redox-sensible mechanisms in postconditioning was shown (Zhao, Corvera 2003). A reduction in superoxide anion generation was observed in the proximity of risk area in postconditioned hearts (Halkos *et al.* 2004, Iliodromitis *et al.* 2006). These findings of reduced ROS production in postconditioning are in line with the idea that massive ROS production is implicated in the sequelae of myocardial reperfusion injury.

Protein kinase C epsilon is a member of group of the PKC family of serine and threonine kinases that are involved in a wide range of physiological processes, including mitogenesis (Yan *et al.* 2013), cell survival under stressful conditions, metastasis (Gorin and Pan 2009) and transcriptional regulation (Lin *et al.* 2011). Thus far, PKCε has been shown to be critical for cardiac protection during H/Re injury (reviewed in (Budas *et al.* 2007). Postconditioning

was associated with significantly higher PKCε levels in areas of the myocardium at risk and selective isoform inhibition prevented the infarct size reduction (Zatta *et al.* 2006). Moreover, the protective effects of ischemic postconditioning were shown to be dependent on PKCε using both the isolated heart model (Philipp *et al.* 2006, Zatta, Kin 2006) and isolated neonatal cardiomyocytes (Dong *et al.* 2010). PKCε is activated by a series of phosphorylations in the catalytic domain at Thr566, Thr710 and Ser729 (Newton 2001). Phosphorylation at Ser729 increases in cardioprotection (Zhou *et al.* 2002) and loss of the Ser729 phosphate is associated with PKCε translocation to the cell periphery or nucleus (Xu *et al.* 2007).

On the basis of these findings, we investigated whether DHS treatment protected cardiomyocytes from simulated hypoxia/reoxygenation injury via a signalling pathway involving PKCε. Western blotting using antibodies to detect the total levels of PKCε and phosphospecific antibodies to detect the Ser 729 phosphorylated PKCε demonstrated that DHS treatment restored the phosphorylated/total PKCε ratio in cardiomyocytes exposed to H/Re (Fig. 7). Following the hypoxia/reoxygenation protocol, our results show a significant decrease in the Ser729–phosphorylated PKCε relative to the total PKCε. Both postconditioning and DHS treatment attenuated such a decrease. These results suggest that both postconditioning and DHS treatment affect the phosphorylation status of PKCε following the hypoxia/reoxygenation in rat primary cardiomyocytes and are consistent with the effects of postconditioning on pPKCε levels observed during myocardial and cerebral ischemia (Gao *et al.* 2008, Zatta, Kin 2006). We conclude that DHS treatment could regulate the activity and phosphorylation status of PKCε protein.

5. Conclusion

Our study demonstrates that DHS treatment reduces cell death in isolated rat neonatal cardiomyocytes after hypoxia/reoxygenation. The attenuation of cellular oxidative stress suggests that this is the main mechanism of the protective activity of DHS. Our data also show that DHS treatment altered the pPKCε/PKCε ratio, which indicates that DHS may mimic the effect of postconditioning by interacting with the PKCε–dependent cardioprotective pathways. The immediacy of the effect and the low micromolar concentration of DHS are promising for a possible pharmacological use. However, additional studies are required to clarify the mechanism operative.

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Abbreviations: DHS: 2,3-dehydrosilybin; DHR: dihydrorhodamine 123; FTSC: fluorescein-5-thiosemicarbazide; H/Re: hypoxia/reoxygenation; PI: propidium iodide; ROS: reactive oxygen species; SB: silybin

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Figures and figure legends

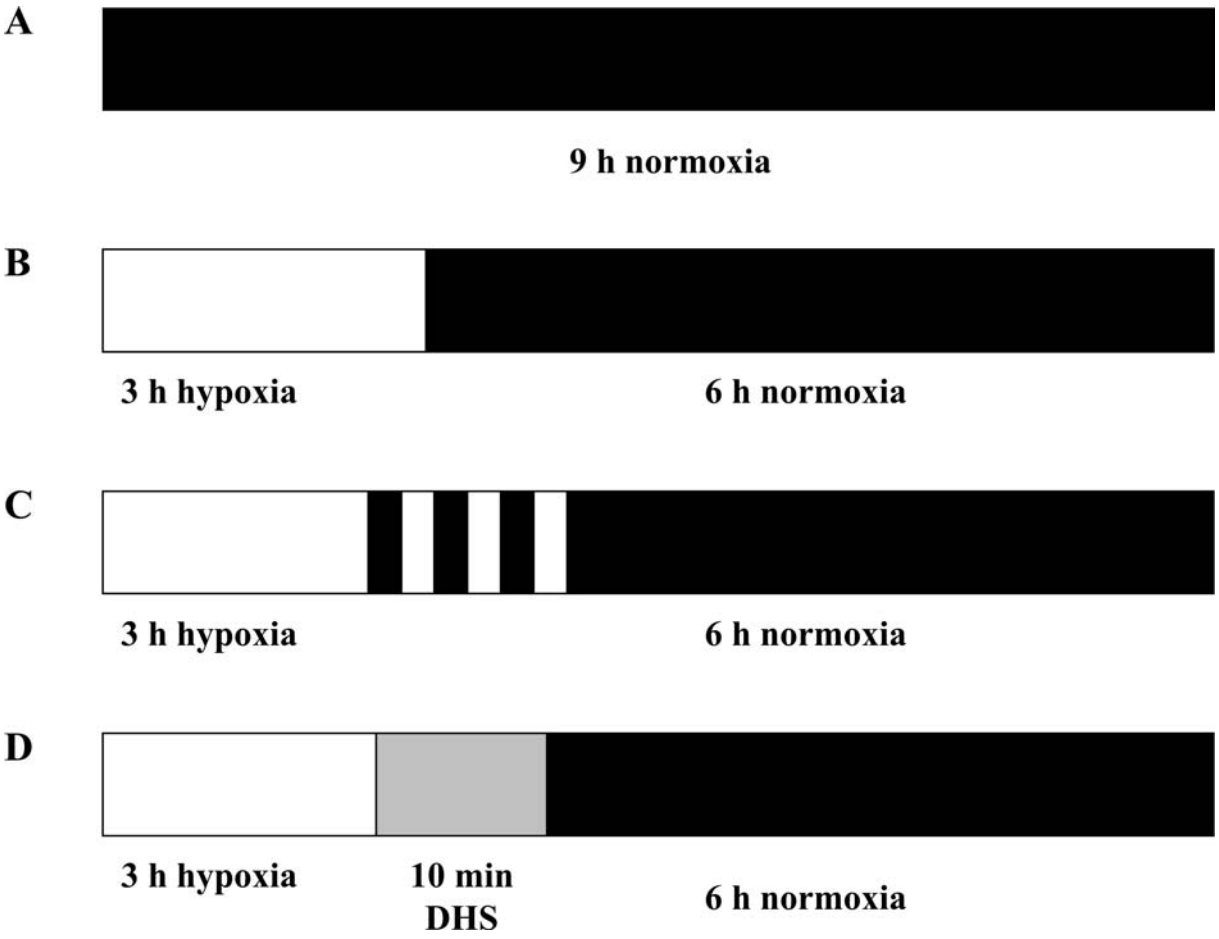

Figure 1. Hypoxia/reoxygenation treatment protocol. Isolated rat cardiomyocytes were divided into four groups. **A.** control group (ctrl), in which cardiomyocytes were incubated for 9 h at normoxic conditions; **B.** hypoxia/reoxygenation (H/Re), in which the culture dishes were transferred to a hypoxic incubator, atmosphere of 1 % O_2, 94 % N_2 and 5 % CO_2 for 3 h followed by 6 h of reoxygenation; **C.** postconditioning (PostC), in which cardiomyocytes were postconditioned after the 3 h index hypoxia by three cycles of 5 min of reoxygenation and 5 min of hypoxia. After the three cycles of postconditioning, 6 h of continuous normoxia was followed. **D.** hypoxia/reoxygenation with 2,3-dehydrosilybin (DHS), in which the culture dishes were transferred to a hypoxic incubator, atmosphere of 1 % O_2, 94 % N_2 and 5 % CO_2 for 3 h followed by 10 min of 10 $\mu mol \cdot l^{-1}$ DHS and 6 h of reoxygenation.

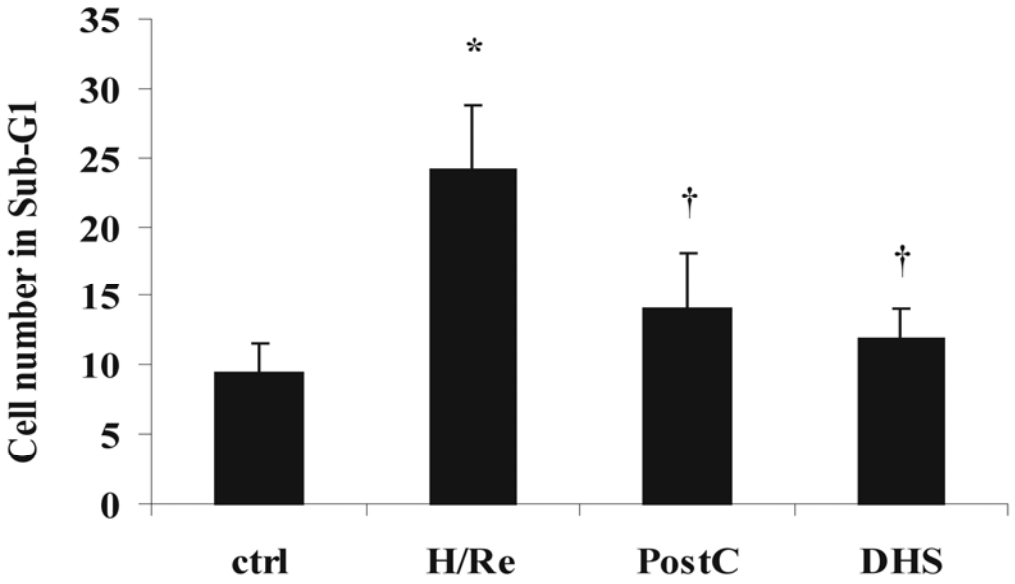

Figure 2. Flow cytometric analysis of cell death in primary rat neonatal cardiomyocytes. Flow cytometric analysis of primary neonatal rat cardiomyocyte death was performed in the control (ctrl), hypoxia/reoxygenation (H/Re), postconditioning (PostC) and hypoxia/reoxygenation with 10 μmol·l^{-1} DHS (DHS) groups. The number of total cell death was determined by propidium iodide staining as mentioned in section Method. The percentageof cells in sub-G1 peak were calculated using MultiCycle AV Software. The data are means ± SEM of four independent experiments. $^{*}p < 0.001$ vs ctrl group, $^{\dagger}p < 0.001$ vs H/Re group.

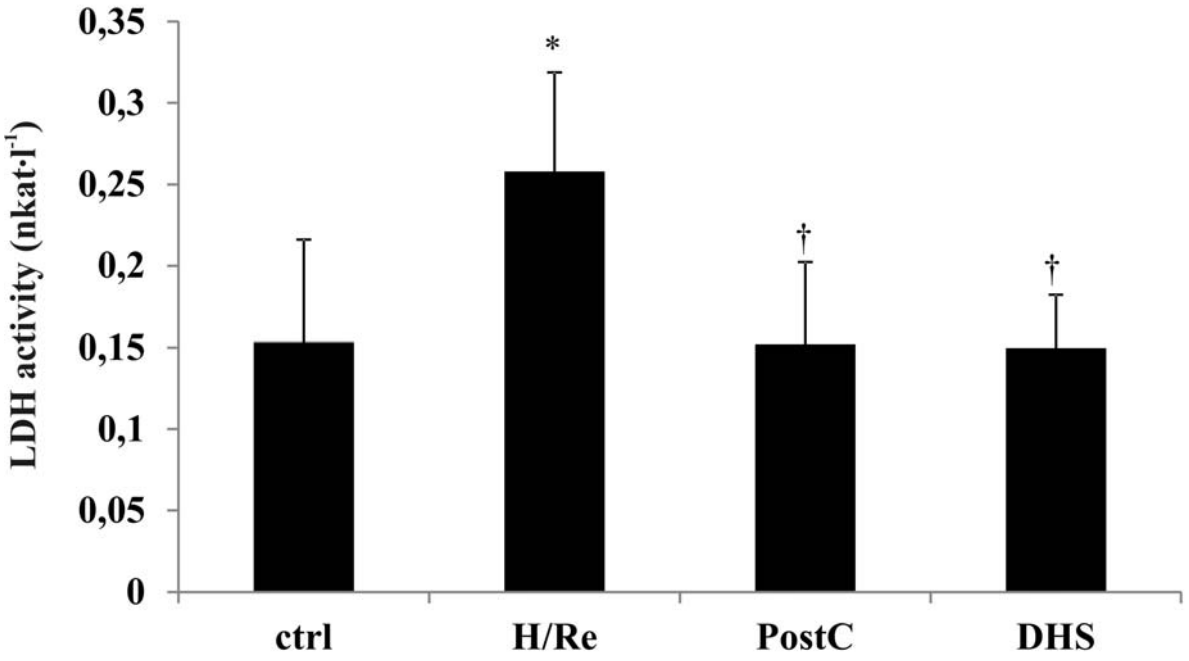

Figure 3. Effect of DHS on LDH leakage in cardiomyocytes exposed to hypoxia/reoxygenation. The activity of lactate dehydrogenase (LDH) released into the culture medium via cell membrane disruption was measured spectrophotometrically at 340 nm in control (ctrl), hypoxia/reoxygenation (H/Re), postconditioning (PostC) and hypoxia/reoxygenation with DHS (DHS) groups. Cell cultures of neonatal rat cardiomyocytes were exposed to DHS (10 μmol·l^{-1}, 10 min) in H/Re group after 3 h of hypoxia. The viability of treated cells was expressed in nkat per liter. The data are means ± SEM of six independent experiments. $^{*}p < 0.001$ vs ctrl group, $^{\dagger}p < 0.001$ vs H/Re group.

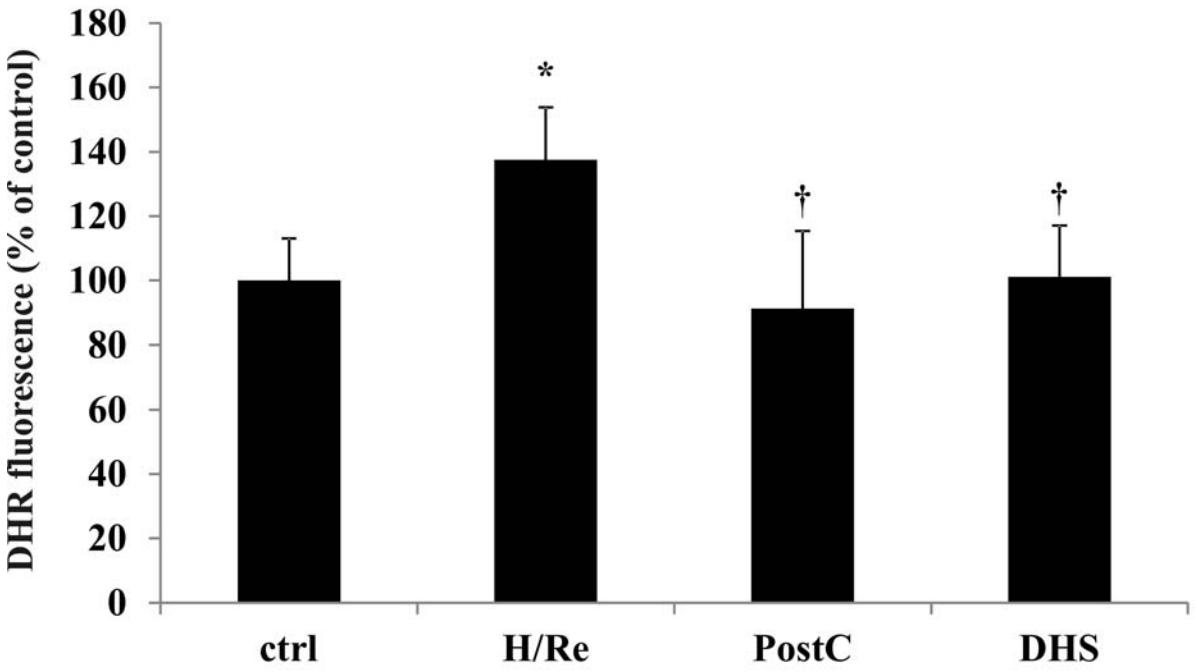

Figure 4. ROS production decreases in hypoxia/reoxygenation group after DHS treatment. Reactive oxygen species (ROS) production was measured using fluorescence probe dihydrorhodamine 123 (DHR) in control (ctrl), hypoxia/reoxygenation (H/Re), postconditioning (PostC) and hypoxia/reoxygenation with DHS (DHS) groups. We have used DHS (10 μmol·l^{-1}, 10 min) for treatment of rat neonatal cardiomyocytes after 3 h of hypoxia. The fluorescence is monitored at specific excitation/emission wavelengths 488/525 nm. The results were expressed as fluorescence of DHR per miligram of protein. The data are means ± SEM of eight independent experiments $^{*}p < 0.001$ vs ctrl group, $^{\dagger}p < 0.001$ vs H/Re group.

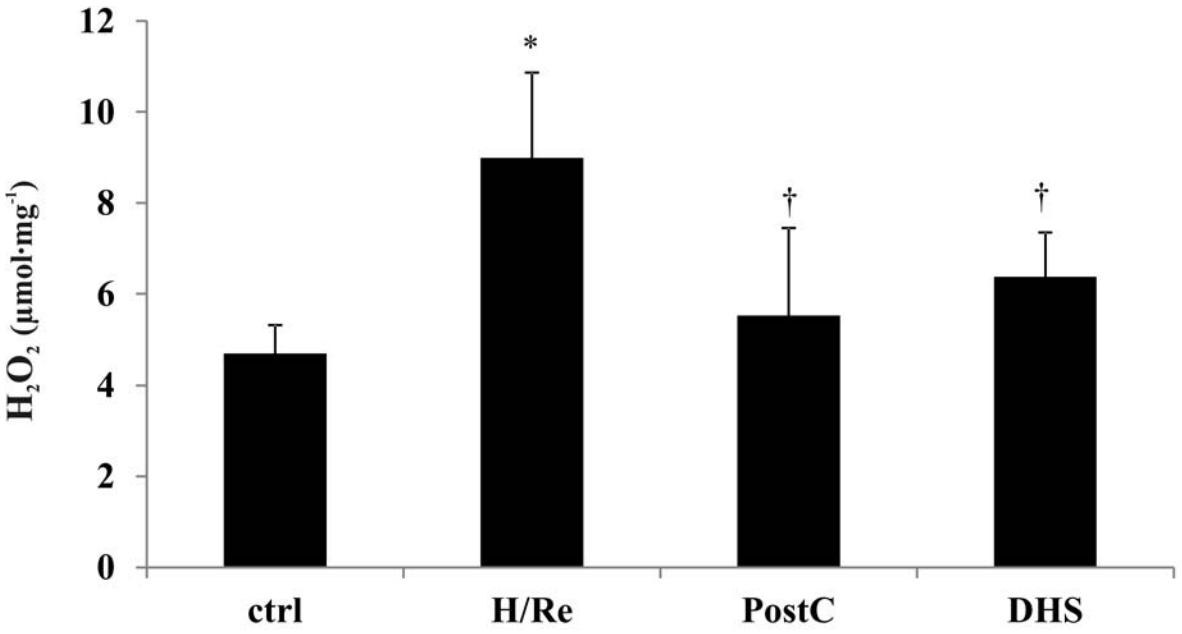

Figure 5. DHS decreases H_2O_2 level in cardiomyocytes exposed to hypoxia/reoxygenation. The H_2O_2 production from cardiomyocytes was determined using the oxidation of the fluorogenic indicator Amplex red in the presence of horseradish peroxidase in control (ctrl), hypoxia/reoxygenation (H/Re), postconditioning (PostC) and hypoxia/reoxygenation with 10 µmol·l^{-1} DHS (DHS) groups. Fluorescence was monitored on a reader with excitation at 570 nm and emission at 585 nm. Micromoles of H_2O_2 were calculated using a standard curve and related to a milligram of protein. The data are means ± SEM of eight independent experiments. $^{*}p < 0.001$ vs ctrl group, $^{\dagger}p < 0.001$ vs H/Re group.

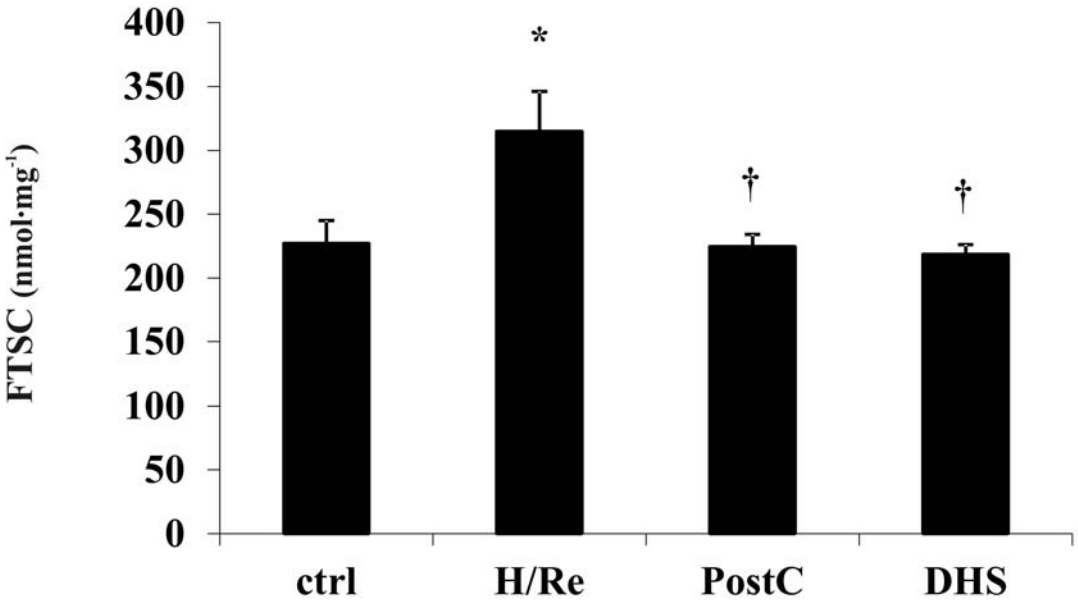

Figure 6. Effect of DHS on protein carbonyls in hypoxia/reoxygenated cardiomyocytes.

Protein carbonyls were detected by fluorescent probe fluorescein-5-thiosemicarbazide (FTSC) in control (ctrl), hypoxia/reoxygenation (H/Re), postconditioning (PostC) and hypoxia/reoxygenation with 10 μmol·l^{-1} DHS (DHS) groups. The fluorescence is monitored at specific excitation/emission wavelengths 485/535 nm. Nanomoles of FTSC-reacted carbonyls were calculated using a standard curve and related to a milligram of protein. The data are means ± SEM of four independent experiments. $^{*}p < 0.001$ vs ctrl group, $^{\dagger}p < 0.001$ vs H/Re group.

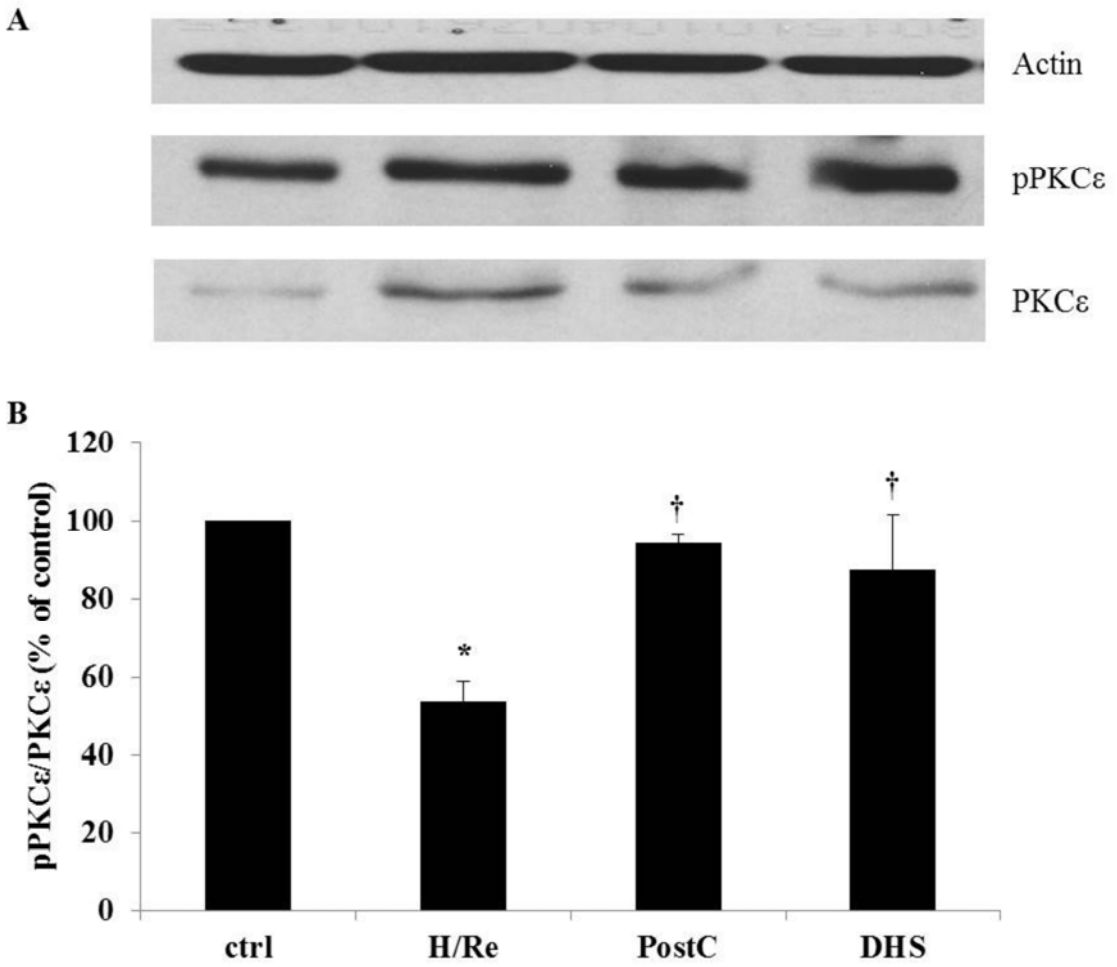

Figure 7. Effect of postconditioning and DHS on protein kinase C epsilon (PKCε) phosphorylation status in rat primary cardiomyocytes after simulated hypoxia/reoxygenation injury.

pPKCε and PKCε protein bands were detected in total cell lysates of control (ctrl), hypoxia/reoxygenation (H/Re), postconditioning (PostC) and hypoxia/reoxygenation with 10 $\mu mol \cdot l^{-1}$ DHS (DHS) groups. Panel A. Representative western blot using total PKCε (PKCε) and Ser 729 phosphospecific (pPKCε) antibodies. Panel B. The intensity of each band was quantified by densitometry, ratio of pPKCε/PKCε was calculated according to the result obtained from scan and data were normalized to the actin signal. The data are means ± SEM of three independent experiments. $^{*}p < 0.001$ vs ctrl group, $^{\dagger}p < 0.001$ vs H/Re group.